Int J Legal Med (2007) 121: 163–168
DOI 10.1007/s00414-006-0087-9

ORIGINAL ARTICLE

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Immunohistochemical investigation of dopaminergic terminal markers and caspase-3 activation in the striatum of human methamphetamine users

Received: 19 December 2005 / Accepted: 2 March 2006 / Published online: 19 April 2006

Abstract Methamphetamine (METH) has been shown to induce neurotoxicity. In a previous human study using quantitative Western blotting and radioligand binding assay, dopaminergic terminal marker deficits were induced in chronic METH users. In this study, we examined the suitability of the immunohistochemical detection of tyrosine hydroxylase (TH), dopamine transporter (DAT), and vesicular monoamine transporter-2 (VMAT2) levels, and caspase-3 activation in the striatum to diagnose METH abuse. Decreases in TH immunoreactivity in the nucleus accumbens and DAT in the nucleus accumbens and putamen were induced in METH users, whereas a significant difference of VMAT2 was not evident between METH and control groups. However, in the nucleus accumbens of two METH users, levels of VMAT2, a stable marker of striatal dopaminergic terminal integrity, were reduced remarkably. These findings might indicate that dopaminergic terminal degeneration is induced in the striatum of some METH abusers. On the other hand, we observed little caspase-3 activation, indicative of apoptosis, in the striatal neurons of chronic METH users. Overall, the findings of dopaminergic terminal markers were similar to those in the previous human study. Therefore, it is suggested that immunohistochemical techniques could be used to examine dopaminergic terminal marker levels and could also give useful information on chronic and/or lethal METH use in cases of METH-related death, where METH intoxication may not be toxicologically demonstrated.

Keywords Methamphetamine · Human brain · Dopaminergic markers · Caspase-3 · Immunohistochemistry

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Introduction

Methamphetamine (METH) is an illicit drug abused worldwide. METH neurotoxicity is characterized by the long-term depletion of striatal monoamines, such as dopamine and serotonin, as well as monoaminergic markers. The METH-induced release of dopamine generates reactive hydrogen species, which are proposed to play an important role in METH neurotoxicity [2, 7]. METH can induce decreases in the immunoreactivity (IR) of dopaminergic terminal markers, such as tyrosine hydroxylase (TH), dopamine transporter (DAT), and vesicular monoamine transporter-2 (VMAT2) in rodents or nonhuman primates [3, 4, 11, 12, 23]. TH is the rate-limiting enzyme in dopamine and noradrenaline synthesis. DAT, a transmembrane protein, removes dopamine from the synapse and returns it to the presynaptic terminal. VMAT2 transports dopamine, serotonin, and norepinephrine from the cytoplasm into specialized secretory vesicles. METH causes monoamine displacement from these vesicles by interacting with VMAT2. VMAT2 is thought to be a stable marker of striatal dopaminergic terminal integrity [11, 25].

In a previous human study, radioligand binding assay and quantitative Western blotting demonstrated dopaminergic terminal marker deficits in chronic METH users [27]; however, these methods are not routinely available for brains from forensic autopsy cases because such brains are very often fixed in formalin or other fixatives for neuropathological examination. To detect alterations in various kinds of protein levels in fixed tissue, immunohistochemistry is widely used, and quantitative analysis of histological images can give useful information on the estimation of human brain damages [13–15].

In addition to dopaminergic marker deficits, other studies have demonstrated METH-induced apoptosis, characterized by distinct morphological changes, such as membrane blebbing, nuclear condensation, and fragmentation of genomic DNA, in the neurons of animals [3, 6, 17]. Caspase-3 has been shown to play the role of executioner most downstream in the apoptotic pathways. Previous

studies have suggested that the detection of neuronal apoptosis after human brain injury could contribute to forensic wound age estimation [8, 14]. To date, there is no evident neuronal loss in areas with decreased dopaminergic marker levels in rodents and nonhuman primates [11, 12]. METH might also injure neurons via apoptosis-independent mechanisms [19]. The question of whether apoptotic mechanisms occur in the striatum of chronic METH abusers remains unsolved.

In this study, we applied immunohistochemical methods to examine dopaminergic terminal marker levels and investigated apoptotic pathways in the striatum of METH users. It has been shown that in situ labeling techniques, such as in situ nick translation or TdT-mediated dUTP nick-end labeling, are useful to demonstrate neuronal apoptosis in human brain injury [14]. In this study, we used an antibody for cleaved caspase-3 to detect apoptotic pathways because immunostaining of cleaved caspase-3 is useful to detect apoptosis in paraffin sections [8, 10], and DNA fragmentation can be induced in necrotic neurons and brain tissue with longer postmortem intervals [1].

Table 1 Information on METH users in this study

Case no.	Sex	Age (years)	Postmortem interval (h)
1	M	44	24
2	M	29	8
3	M	42	19
4	M	24	23
5	M	34	13
6	F	31	21
7	M	22	24
8	M	23	12
9	M	45	24
10	F	18	19
11	M	29	10

M male, *F* female
Blood METH levels: 0.5–17.0 mg/ml (cases 1–10), negative (case 11)
Urine METH levels: 0.4–43.5 mg/ml (cases 2–6, 8, 9, 11), not available (cases 1, 7, 10)

Materials and methods

Cases

Brain samples from 11 chronic METH users (nine males, two females; mean age, 31 years old) and 11 drug-free control subjects (seven males, four females; mean age, 32.7 years old) were examined. METH cases are summarized in Table 1. To select cases, toxicological screening was tested with Triage (Biotest, San Diego, USA) and the quantitative analysis described later. The control group had no history of METH use and tested negative for METH and other recreational drugs in the urine and/or blood. In this study, we selected METH users whose cause of death was acute methamphetamine intoxication because they might have self-administered enough METH to induce oxidative damage. METH was detected in both blood and urine samples in cases 1–10 and only in urine in case 11. We could not obtain detailed drug histories from the METH users because of a lack of reliable information in most cases. Hair samples from all METH users were METH-positive, indicating that all METH users in this study were chronic drug users. The causes of death of the control subjects were bleeding (n=7), asphyxia (n=2), and ischemic heart disease (n=2).

METH quantitation by gas chromatography/mass spectroscopy

Hair samples were collected in 1-cm segments relative to the root, washed in distilled water, and dissolved in 10% NaOH overnight. The dissolved samples were extracted with *n*-hexane. Samples of blood and urine were added to sodium borate and extracted with ethyl acetate. The extracts of hair, blood, and urine samples were dried after acidifying with one drop of acetic acid. The dried extracts were dissolved in ethyl acetate, derivatized with trichloroacetic anhydride and analyzed with gas chromatography/mass spectroscopy (GC/MS).

GC/MS was performed on a Shimadzu GCMS-QP5050A (Kyoto, Japan) and operated in electron impact mode at 70 eV. A DB-1 column (30 m by 0.53 mm inner diameter and 1.5 mm film thickness), obtained from J&W Scientific (Folsom, CA, USA), was run using helium as the carrier gas at a flow rate of 8 ml/m. The injector temperature was 250°C, and the GC/MS interface temperature was 230°C. The initial temperature was 80°C for 5 min, followed by an increase of 10°C/min to 200°C.

Immunohistochemical staining

Brains were fixed in buffered formalin fixative. The nucleus accumbens, putamen, and caudate were embedded in paraffin and cut into 6-μm thick sections. After deparaffinization, the sections were incubated with anti-TH (monoclonal, mouse, 1:1,000, Chemicon International, Temecula, CA), anti-DAT (polyclonal, rabbit, 1:100, Chemicon International), anti-VMAT2 (polyclonal, rabbit, 1:200, Chemicon International), and anti-cleaved caspase-3 (polyclonal, rabbit, 1:200, Cell Signaling Technologies, Beverly, MA) antibodies. After incubation of primary antibodies, the DAKO ENVISION System (DakoCytomation, Kyoto, Japan) was used for incubation with primary antibodies and visualization with 3-3′-diaminobenzidine tetrahydrochloride. Before immunostaining, the sections were pretreated in 0.01 M citrate buffer, pH 6.0, with hydrated autoclaving for 10 min. As a positive control for cleaved caspase-3 immunostaining, we used brain tissue with ischemic damage from a 67-year-old female.

Neuron-specific enolase (NSE), a neuronal marker, was not affected in chronic METH users [27], so we immuno-

stained the protein using anti-NSE antibody (monoclonal, mouse, no dilution, DakoCytomation) as a primary antibody and then visualized the sections as described above. There was no significant difference of perikaryal NSE-IR between both groups ($p>0.05$, data not shown).

For the measurement of dopaminergic marker levels, images were captured by a digital camera (DXM1200F, Nikon, Tokyo, Japan) connected to a microscope (Eclipse 80i, Nikon) with an objective ×20. The images were transformed into 8-bit grayscale files on Adobe Photoshop 7.0 and analyzed using ImageJ 1.34s software (NIH, USA), as previously reported [26]. Briefly, digitalized images were thresholded in ImageJ using the threshold tool, with visual comparison made to the original grayscale images. Thereafter, particle analysis for each image was carried out using the analyze particle tool.

Statistical analysis was by unpaired *t* test. Differences were considered statistically significant at $p<0.05$.

Results

Dopaminergic terminal markers

TH immunohistochemistry in the nucleus accumbens showed decreases in TH-IR in the METH group compared with control subjects (Fig. 2a,b). Statistical analysis revealed a significant decrease in TH-IR in the nucleus accumbens (−53%, $p<0.01$), although there were no significant differences in the other two subdivisions (Fig. 1a).

DAT was immunostained densely and heterogeneously in the striatum (Fig. 2c), as previously reported [5], and decreased DAT-IR was observed in METH users (Fig. 2d). There were remarkable reductions in DAT-IR in the nucleus accumbens and putamen (−60%, $p<0.05$; −59%, $p<0.05$, respectively) of METH cases, and the DAT level in the caudate showed a reduction (−21%), but not significantly ($p>0.05$)(Fig. 1b).

Although VMAT2 in the nucleus accumbens and putamen of the METH group reduced by 31 and 29% of the control, respectively, but not significantly ($p>0.05$) (Fig. 1c), image analysis in individual cases revealed that the nucleus accumbens of two METH cases (cases 10 and 11) showed over 90% reduction in VMAT2-IR, accompanied by profound decreases in TH-IR (−96 and −76%, respectively) and DAT-IR (−92% and −96%, respectively). The immunostained images of a control subject and case 11 are shown in Fig. 2e,f.

Caspase-3

We employed cleaved caspase-3 as an apoptotic marker in this study. In the ischemic brain of a 67-year-old female, immunostaining revealed cytoplasmic localization of cleaved caspase-3 in some neurons (Fig. 2g), whereas caspase-3 activation was not evident in the striatum of any METH users (Fig. 2h) or in the control group.

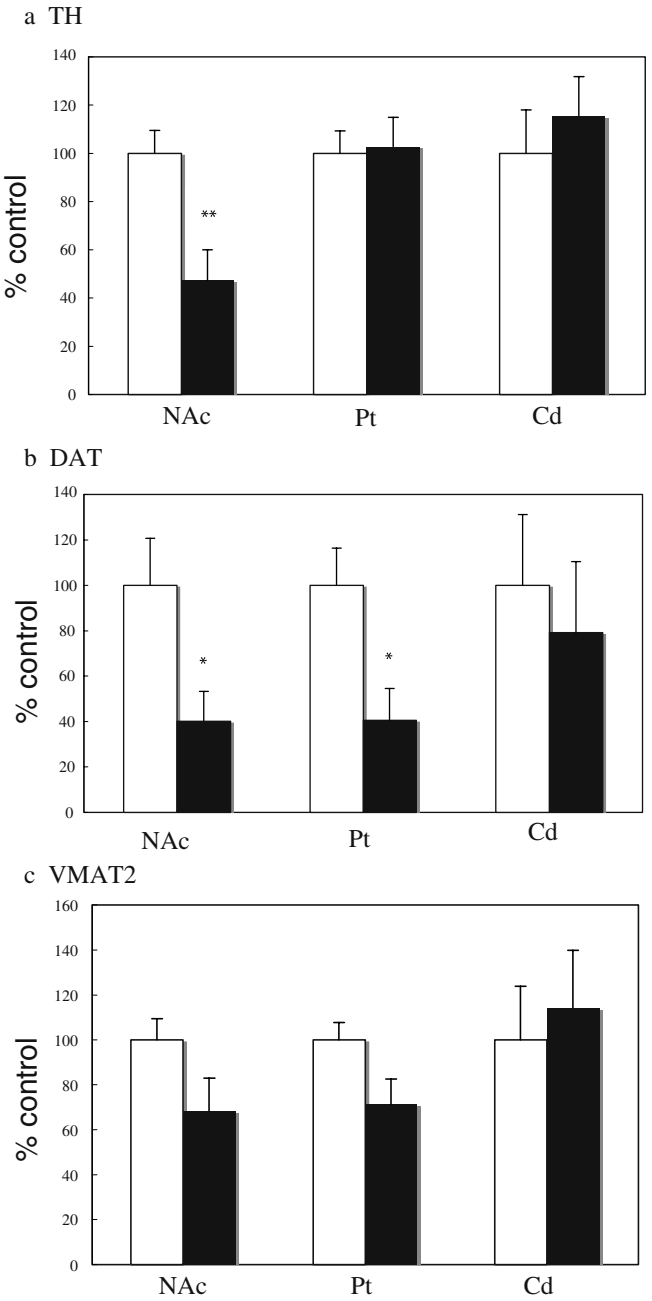

Fig. 1 Quantitative analysis of dopaminergic terminal marker levels in the striatum of control (*openbars*) and METH groups (*filledbars*). **a** TH, **b** DAT, **c** VMAT2. Data are expressed as the mean values ±SEM (*bars*) percentages of control. $**p<0.01$; $*p<0.05$, significantly different from the control group. *NAc* Nucleus accumbens, *Pt* putamen, *Cd* caudate

Discussion

Dopaminergic terminal markers

In this study, immunohistochemical staining demonstrated significant decreases in TH-IR in the nucleus accumbens.

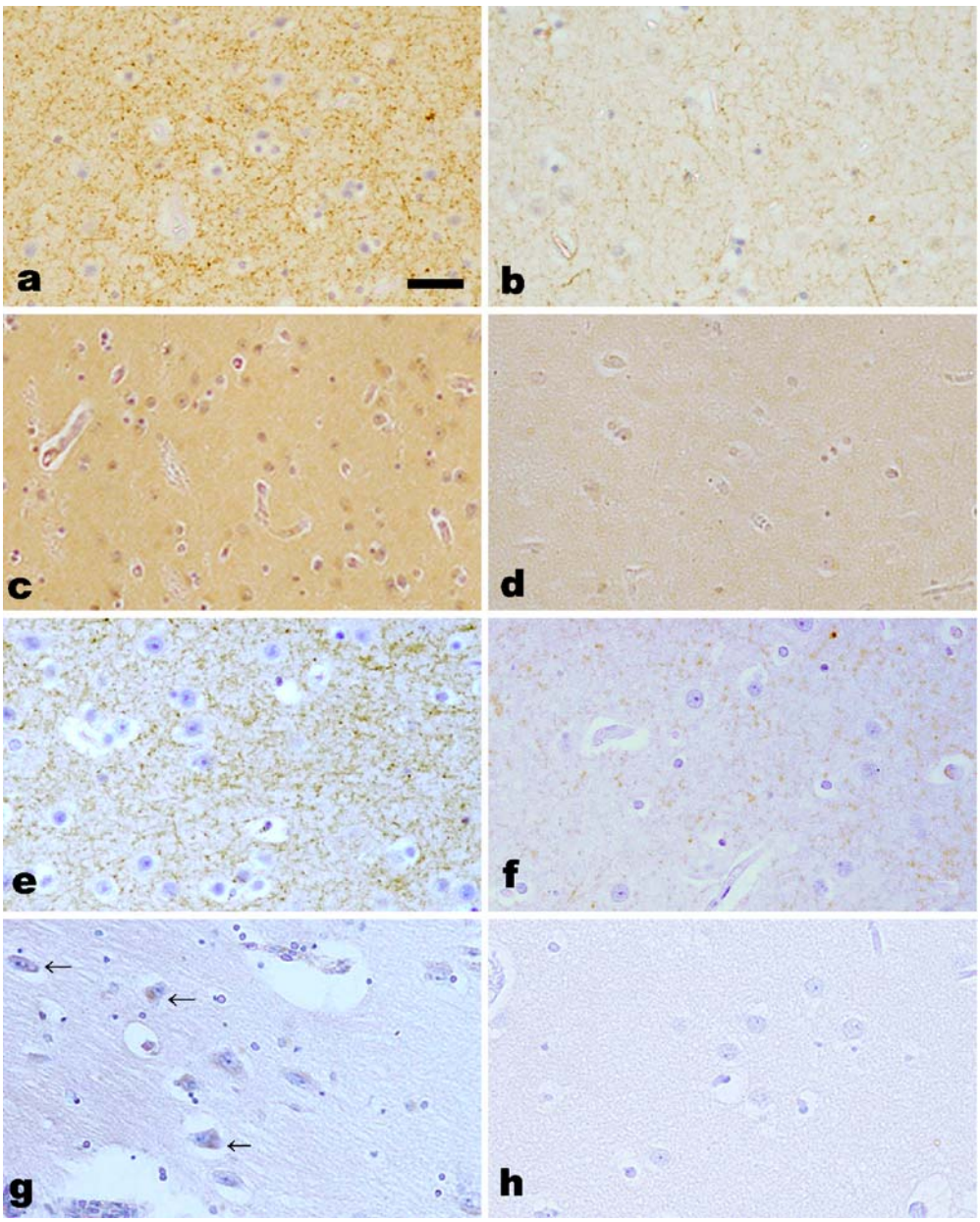

Fig. 2 Immunostaining of TH (**a**, **b**), DAT (**c**, **d**), VMAT2 (**e**, **f**), and cleaved caspase-3 (**g**, **h**) in the nucleus accumbens. Remarkable decreases in dopaminergic terminal marker-IR were observed in case 11 (**b**, **d**, **f**) compared with the brain of a control subject (29-year-old male who died of bleeding) (**a**, **c**, **e**). Cleaved caspase-3 was observed in the cytoplasm of neurons (*arrows*) of a 67-year-old female with brain ischemia (**g**). On the other hand, caspase-3 activation was not evident in the nucleus accumbens with dopaminergic terminal marker deficits of case 11 (**h**). Bar, 50 μm

There were significant decreases in DAT-IR in the nucleus accumbens and putamen of METH users, and the DAT level in the caudate was reduced, but not significantly. On the other hand, Western blotting detected a decrease in DAT levels in the caudate, as well as DAT reductions in the nucleus accumbens and putamen [27]. However, DAT reduction in the caudate was less remarkable than in other striatal subdivisions, and one of two radioactive ligand bindings was normal [27]. Overall, our findings were similar to the previous study. Therefore, immunohistochemical techniques could be suitable to detect dopaminergic terminal marker deficits in forensic autopsy cases.

Different to TH and DAT levels, a significant decrease in VMAT2 level was not evident in the METH group, and our results are consistent with previous findings in human METH users [27], supporting the notion that VMAT2, a stable marker of striatal dopaminergic terminal integrity, might not be a sensitive marker of METH neurotoxicity [11, 25]; however, our study demonstrated that VMAT2-IR, together with TH-IR and DAT-IR, was remarkably decreased in the nucleus accumbens of two METH users (cases 10 and 11), suggesting that dopaminergic terminal degeneration is induced in the striatum of some METH abusers. One of the METH cases (case 11) might have survived more than 24 h after a high dose of METH administration, because METH was negative in the blood sample. Prior studies in rodents revealed decreased VMAT2 levels 6 days after neurotoxic administration regimes of METH [9, 16], indicating that VMAT2 levels in the striatum fall long-term after high-dose METH administration. Taken together with our findings and with that of previous studies, VMAT2 deficits in the striatum might be indicative of long-term survival after METH administration.

Another METH case (case 10) with dopaminergic marker deficits might have died within 24 h after lethal METH exposure because METH was positive in the blood and urine, suggesting other factors might affect dopaminergic marker levels. The repeated administration of METH at low doses, which is similar to the patterns of recreational use, blocked the depletion of dopamine, serotonin, and their metabolites induced by toxic doses of METH [18, 24]. In addition, the activity of the enzyme copper–zinc superoxide dismutase, responsible for the conversion of superoxide to hydrogen peroxide and molecular oxygen, was induced in chronic METH users [22]. On the basis of these previous investigations, protective mechanisms against METH-induced dopaminergic terminal degeneration might be induced to some extent by recreational METH use; however, the extent of dopaminergic marker deficits might be exacerbated when such mechanisms are not induced.

Caspase-3 activation

The lack of caspase-3 activation in the striatum containing dopaminergic marker deficits in this study failed to confirm the involvement of apoptotic mechanisms in METH neurotoxicity of human abusers. Previous studies demonstrated caspase-3 activation and DNA fragmentation in METH-treated animals and cultured cells with severe dopaminergic terminal marker deficits [3, 6, 17]. A recent study revealed that a regime of escalating doses of METH suppressed hyperthermia induced by toxic doses of the drug [24]. In addition, brain-derived neuronal factor, which is induced in rat brains by repeated low-dose amphetamine administration [21], inhibits caspase-3 activation and increases neuronal viability [20]. These findings suggest that the repeated administration of METH might inhibit or decelerate caspase-3 activation in METH users. On the other hand, it has been suggested that METH induces neuronal damage via apoptosis-independent mechanisms [19]. Further studies will be needed to establish neuronal changes in METH users.

Conclusion

We failed to detect immunohistochemical evidence of caspase-3 in the striatum of METH users; however, we demonstrated that immunohistochemistry could detect the dopaminergic terminal marker in METH users, and these findings might be useful in diagnosing METH-related death in cases where little or low METH is detected toxicologically in postmortem samples because of longer survival periods after lethal METH use.

Acknowledgement This work was supported by grants-in-aid for General Scientific Research (17590579) from the Ministry of Education, Science, Sports and Culture of Japan.

References

1. Anderson AJ, Su JH, Cotman CW (1996) DNA damage and apoptosis in Alzheimer's disease: colocalization with c-Jun immunoreactivity, relationship to brain area and effect of postmortem delay. J Neurosci 16:1710–1719
2. Cadet JL, Brannock C (1998) Free radicals and the pathology of brain dopamine systems. Neurochem Int 32:117–131
3. Cadet JL, Jayanthi S, Deng X (2003) Speed kills: cellular and molecular bases of methamphetamine-induced nerve terminal degeneration and neuronal apoptosis. FASEB J 17:1775–1788
4. Cappon GD, Pu C, Vorhees CV (2000) Time-course of methamphetamine-induced neurotoxicity in rat caudate–putamen after single dose treatment. Brain Res 863:106–111
5. Ciliax BJ, Drash GW, Staley JK, Haber S, Mobley CJ, Miller GW, Mufson EJ, Mash DC, Levey AI (1999) Immunocytochemical localization of the dopamine transporter in human brain. J Comp Neurol 409:38–56
6. Deng X, Wang Y, Chou J, Cadet JL (2001) Methamphetamine causes widespread apoptosis in the mouse brain: evidence from using an improved TUNEL histochemical method. Brain Res Mol Brain Res 93:64–69
7. De Vito MJ, Wagner GC (1989) Methamphetamine-induced neuronal damage: a possible role for free radicals. Neuropharmacology 28:1145–1150
8. Dreßler J, Hanisch U, Busuttil A (2005) Comments on Hausmann et al.: neuronal apoptosis following human brain injury. Int J Legal Med 119:177–178
9. Frey K, Kilbourn M, Robinson T (1997) Reduced striatal vesicular monoamine transporters after neurotoxic but not after behaviorally-sensitizing doses of methamphetamine. Eur J Pharmacol 334:273–279
10. Gown AM, Willingham MC (2002) Improved detection of apoptotic cells in archival paraffin sections: immunohistochemistry using antibodies to cleaved caspase 3. J Histochem Cytochem 50:449–454
11. Guilarte TR, Nihei MK, McGlothan JL, Howard AS (2003) Methamphetamine-induced deficits of monoaminergic neuronal markers: distal axotomy or neuronal plasticity. Neuroscience 122:499–513
12. Harvey DC, Lacan G, Tanious SP, Melega WP (2000) Recovery from methamphetamine-induced long-term nigrostriatal dopaminergic deficits without substantia nigra cell loss. Brain Res 871:259–270
13. Hausmann R, Kaiser A, Lang C, Bohnert M, Betz P (1999) Quantitative immunohistochemical study on the time-dependent course of acute inflammatory cellular response to human brain injury. Int J Legal Med 112:227–232
14. Hausmann R, Biermann T, Wiest I, Tübel J, Betz P (2004) Neuronal apoptosis following brain injury. Int J Legal Med 118:32–36
15. Hausmann R, Vogel C, Seidl S, Betz P (2006) Value of morphological parameters for grading of brain swelling. Int J Legal Med (in press). DOI 10.1007/s00414-005-0021-6
16. Hogan KA, Staal RGW, Sonsalla PK (2000) Analysis of VMAT2 binding after methamphetamine or MPTP treatment: disparity between homogenates and vesicular preparations. J Neurochem 74:2217–2220
17. Jayanthi S, Deng X, Noailles P-AH, Ladenheim B, Cadet JL (2004) Methamphetamine induces neuronal apoptosis via cross-talks between endoplasmic reticulum and mitochondrial-dependent death cascades. FASEB J 18:238–251
18. Johnson-Davis KL, Fleckenstein AE, Wilkins DG (2003) The role of hyperthermia and metabolism as mechanisms of tolerance to methamphetamine neurotoxicity. Eur J Pharmacol 482:151–154
19. Larsen KE, Fon EA, Hastings TG, Edwards RH, Sulzer D (2002) Methamphetamine-induced degeneration of dopaminergic neurons involves autophagy and upregulation of dopamine synthesis. J Neurosci 15:8951–8960

20. Matsuzaki H, Namikawa K, Kiyama H, Mori N, Sato K (2004) Brain-derived neurotrophic factor rescues neuronal death induced by methamphetamine. Biol Psychiatry 55:52–60
21. Meredith GE, Callen S, Scheuer DA (2002) Brain-derived neurotrophic factor expression in the rat amygdala, piriform cortex and hypothalamus following repeated amphetamine administration. Brain Res 949:218–227
22. Mirecki A, Fitzmaurice P, Ang L, Kalasinski KS, Peretti FJ, Aiken SS, Wickham DJ, Sherwin A, Nobrega JN, Forman HJ, Kish SJ (2004) Brain antioxidant systems in human methamphetamine users. J Neurochem 89:1396–1408
23. O'Callaghan JP, Miller B (1994) Neurotoxicity profiles of substituted amphetamine in the C57BL/6J mouse. J Pharmacol Exp Ther 270:741–751
24. Segal DS, Kuczenski R, O'Neil ML, Melega WP, Cho AK (2003) Escalating dose methamphetamine pretreatment alters the behavioral and neurochemical profiles associated with exposure to a high-dose methamphetamine binge. Neuropsychopharmacology 28:1730–1740
25. Vander Borght TM, Kilbourn MR, Desmond T, Kuhl D, Frey K (1995) The vesicular monoamine transporter is not regulated by dopaminergic drug treatments. Eur J Pharmacol 294:577–583
26. Williams SM, Bryan-Lluka LJ, Pow DV (2005) Quantitative analysis of immunolabeling for serotonin and for glutamate transporter after administration of imipramine and citalopram. Brain Res 1042:224–232
27. Wilson JM, Kalasinski KS, Levey AI, Bergeron C, Reiber G, Anthony RT, Schmunk GA, Shannak K, Haycock JW, Kish SJ (1996) Striatal dopamine nerve terminal markers in human, chronic methamphetamine users. Nat Med 2:699–703